

Identification of the U-937 membrane-associated proteinase interacting with the V3 loop of HIV-1 gp120 as cathepsin G

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Abstract

We have purified a serine proteinase from the membrane of U-937 cells that was inhibited in a tight-binding manner by recombinant gp120 and by peptides mimicking the V3 loop of gp120 [(1993) FEBS Lett. 317, 167–172]. This proteinase has now been characterized, both structurally and functionally. It has a dual trypsin- and chymotrypsin-like specificity, and N-terminal sequence analysis of the first 32 residues indicates complete identity with leukocyte cathepsin G. Cathepsin G-like material was located at the surface of U-937 cells using a monoclonal antibody directed against leukocyte cathepsin G, and polyclonal anti-cathepsin G antibodies precipitated the purified proteinase. However, the U-937 enzyme differs slightly from commercial leukocyte cathepsin G in its apparent M_r because of different glycosylation. No other protein structurally related to cathepsin G was found upon screening a U-937 cDNA library using several oligonucleotide probes constructed from the membrane proteinase N-terminal amino acid sequence. The possible interaction of a cathepsin G-like proteinase at the surface of U-937 cells with the V3 loop of HIV-1 gp120 is discussed.

Key words: HIV-1; gp120 V3 loop; Proteinase; Cathepsin G

1. Introduction

The binding of the gp120 envelope glycoprotein of HIV-1 to the CD4 receptor of permissive cells does not seem to be sufficient to allow virus entry. The possibility that additional components at the surface of host cells are required for cell infection has been widely discussed [1–5]. The identity of these components is not yet known, but they might be cell-specific and also differ from species to species [6,7]. It has been suggested that one of these cofactors could be a proteolytic enzyme, because a conserved region at the crown of the V3 loop is similar to Kunitz-type proteinase inhibitors [8]. A membrane-associated proteinase that interacts with the V3 loop of HIV-1 gp120 was first identified on the CD4⁺ T lymphocyte membrane by Hattori et al. [9,10]. Since then, other membrane proteinases that interact with the V3 loop or may be involved in HIV infection have been described at the surface of HIV-sensitive cell lines [8–13]; the most recently reported is dipeptidyl peptidase IV [14]. Shultz et al. [15] have suggested that cell surface proteinases cleave gp120, allowing a change in conformation necessary for virus–host cell fusion. Several sites within the V3

loop of different HIV strains that could be cleaved by trypsin-like, chymotrypsin-like and aspartyl proteinases have been identified [16], but no proof has been provided that gp120 is actually cleaved during infection. It could be that the simple binding of gp120 to the surface proteinase via its V3 loop, without subsequent cleavage, is sufficient for correct exposure of the virus, fusion, and entry into the cell. Whether or not the cleavage of the V3 loop is required in the process, the proteinase that may be involved should have a rather wide specificity if one takes into account the variability of V3 sequences among infectious HIV isolates [15]. Interestingly, tryptase TL-2 has both trypsin-like and chymotrypsin-like specificities, which satisfy this requirement [9]. Small changes in the amino acid sequence of the V3 loop can result in dramatic changes in cell tropism, cytopathogenicity and infection [4,17,18]. This is consistent with the role of a proteinase as a cofactor, the specificity of which may be abolished by a single amino acid substitution at a critical position in the substrate [19].

We have previously shown that a membrane-associated serine proteinase from U-937 cells interacts and is specifically inhibited by recombinant gp120 from HIV-1 and by synthetic peptides spanning the V3 loop [13]. However, the small amount of material obtained after the last step of purification was not sufficient for its complete physicochemical characterization or subsequent investigation of its potential role in HIV infection. This report further describes the physicochemistry and function of the proteinase. The results are discussed in terms of its possible involvement in infection by HIV.

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Abbreviations: ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); DTNB, (5,5'-dithiobis-(2-nitrobenzoic acid)); NaCl/P_i, phosphate-buffered saline; PCR, polymerase chain reaction; STI, soybean trypsin inhibitor; Suc-Ala-Ala-Pro-Phe-SBzl, *N*- α -Cbz-alanyl-alanyl-prolyl-phenylalanyl-thiobenzylester · HCl; TFA, trifluoroacetic acid; Z-Lys-SBzl, *N*- α -Cbz-L-Lysine-thiobenzylester · HCl.

2. Materials and methods

2.1. Purification and identification of a membrane-associated serine proteinase from U-937 monocytes

Cells from the human promonocyte cell line U-937 [20] were cultured as described earlier [13]. Briefly about $2 \cdot 10^{10}$ cells were resuspended in relax buffer (10 mM piperazine-*N,N'*-bis(2-ethane sulfonic acid) HCl, pH 6.8, 3.5 mM $MgCl_2$, 0.1 M KCl, 1.25 mM ethylenediaminetetraacetic acid) and disrupted by N_2 cavitation. The resulting lysate was centrifuged at $5,000 \times g$ (20 min, $4^\circ C$) to eliminate nuclei and intact cells. Granules and mitochondria were pelleted by centrifugation at $45,000 \times g$ for 13 min at $4^\circ C$. Finally, membranes were isolated by ultracentrifugation ($100,000 \times g$, 60 min, $4^\circ C$) and their purity checked by electron microscopy. The membrane pellet was extracted twice by sonication and centrifuged ($25,000 \times g$, 20 min, $4^\circ C$). Supernatants were layered on agarose-immobilized benzamidine and eluted as in [13]. The following steps of chromatography on hydroxyapatite and Superose 12 remained unchanged. All the fractions eluted from the Superose 12 column were assayed for esterolytic activity using Z-Lys-SBzl and Suc-Ala-Ala-Pro-Phe-SBzl as substrates. Enzymatically active fractions were pooled, concentrated, titrated with STI and stored at $-70^\circ C$ in 5 mM sodium phosphate buffer, 2 M NaCl, pH 6.2.

2.2. Enzyme assays

The chromogenic substrates Z-Lys-SBzl and Suc-Ala-Ala-Pro-Phe-SBzl were assayed under standardized conditions [13,21,22]. Kinetic parameters were determined by varying the amount of substrate (0.25–1.125 mM) and using the Hanes linear plot (S/v vs. S) [23]. The purified membrane proteinase was titrated with STI, prepared as a $3.5 \cdot 10^{-7}$ M stock solution in 0.1 M Tris-HCl buffer, pH 8.2 [24]. Purified enzyme (5 μ l) was incubated with inhibitor (0 – $1.2 \cdot 10^{-8}$ M final concentration) for 15 min at $25^\circ C$ before starting the reaction with Z-Lys-SBzl or Suc-Ala-Ala-Pro-Phe-SBzl (0.37 mM final). Substrate hydrolysis was measured at 412 nm (200 μ l final volume) on a microplate reader (Thermomax, Molecular Devices).

Kinetic parameters were determined and commercial cathepsin G (ICN Biochemicals) titrated as reported above.

The synthetic peptide R13K (RKSIRIQRGPRK), corresponding to fragment 309–320 of the V3 loop sequence of HIV-1 *Lai* was synthesized and purified [13]. Its inhibitory properties were assessed by incubation with U-937 proteinase or commercial cathepsin G (9 nM final concentrations) for 15 min at $25^\circ C$, and measurement of the remaining activity as reported above.

2.3. Amino-terminal sequence

Purified proteinase (Superose 12) was reduced, pyridylethylated and fractionated by reverse-phase chromatography on a C4 cartridge using an isopropanol gradient (0–85%) in 0.1% formic acid. Eluted material was concentrated on a ProSpin membrane (Applied Biosystems) which was washed three times in 20% methanol. About 100 pmol of sample was used for N-terminal sequencing in an Applied Biosystems 477A protein sequencer. Phenylthiohydantoin derivatives were identified on an on-line associated ABI 120A analyser [25].

2.4. Deglycosylation by endo- β -N-acetyl glucosaminidase F

Purified proteinase from U-937 membranes and commercial cathepsin G were deglycosylated using endo- β -N-acetyl glucosaminidase F (endo-F; Boehringer) at a maximal concentration of 2 U/mg protein. Mixtures were incubated overnight at $30^\circ C$ in 50 mM potassium phosphate, pH 7.4, 25 mM EDTA, 0.5% Triton X-100, 0.2% SDS, 1% 2-mercaptoethanol, and analysed by SDS gel-electrophoresis and immunoblotting using the PhastSystem (Pharmacia).

2.5. Immunoassays

2.5.1. Immunoblotting. SDS-PAGE (12.5%) was performed using the PhastSystem (Pharmacia) by the procedure of Laemmli [26]. Proteins were transferred to nitrocellulose (0.45 μ m; Sartorius) by semi-dry electrotransfer for 15 min at 25 mA/20 V. The nitrocellulose sheet was blocked with NaCl/P_i (10 mM phosphate, pH 7.4, 150 mM NaCl) containing 5% dried milk (w/v) at $37^\circ C$ for 120 min and treated as described elsewhere [27]. Anticathepsin G antibodies (ICN Biochemicals) (diluted 1/500 in NaCl/P_i, 0.1% Tween 20, 5% dried milk) were added and incubated for 18 h at $4^\circ C$. The membrane was then washed three times with NaCl/P_i containing 0.05% Tween 20 and incubated

with anti-sheep IgG-peroxidase conjugate (Sigma) (diluted 1/500 in NaCl/P_i, 0.05% Tween 20, 1% dried milk) for 2 h at room temperature and washed as before. Antigen was detected by chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

2.5.2. Antibody binding assay (ELISA test). Microtitration plates (Maxisorp; Nunc) were coated with 100 μ l of commercial cathepsin G (ICN Biochemicals), trypsin (Boehringer), kallikrein (rK10, a generous gift of Dr. N. Gutman) or membrane-associated serine proteinase from U-937 (0.5 mg/ml 0.1 M Tris-HCl buffer, pH 8.2, overnight at $37^\circ C$). The plates were washed once with NaCl/P_i, 0.1% Tween 20, and saturated with NaCl/P_i, 10% dried milk for 30 min at $37^\circ C$ (200 μ l/well). Anti-cathepsin G antibodies (diluted 1/500 in NaCl/P_i containing 2.5% dried milk) were added and incubated for 1 h at $37^\circ C$. Plates were then washed three times with NaCl/P_i, 0.1% Tween 20, and 100 μ l of peroxidase-conjugated donkey anti-sheep IgG (Sigma) (diluted 1/1,000) were added to each well and incubated for 1 h at $37^\circ C$. Plates were washed again, and wells were filled with 100 μ l ABTS containing 0.03% H_2O_2 in 0.1 M citrate-sodium phosphate buffer, pH 4.5, and incubated for 15 min in the dark. Absorbance was read at 405 nm (Thermomax Molecular Devices).

2.5.3. Fluorescence analysis. U937 cells were washed in PBS, 3% foetal calf serum, and labeled with anti-cathepsin G (Mab 1054; Euromedex) or anti-CD4 (OKT4.A; Ortho-Diagnostics Systems) monoclonal antibodies. Upon incubation and washing, antibody binding was evaluated by flow cytometry (Facs-star; Becton-Dickinson) after incubation with FITC-conjugated anti-mouse Ig antibodies (Becton-Dickinson).

3. Results

The early steps of the purification procedure previously used to isolate a serine proteinase interacting with HIV-1 gp120 and peptides of the V3 loop from the membrane fraction of U-937 cells were modified as described in section 2. The main change was in the preparation of the membrane extract, which resulted in a ten-fold increase in material with serine proteinase activity. This activity was fractionated on immobilized benzamidine which retained the minor amount of serine proteinase activity inhibited by peptides of the V3 loop of HIV 1 under the chosen experimental conditions. This material was chromatographed on hydroxyapatite and Superose 12 [13] yielding about 220 μ g of serine proteinase from $2 \cdot 10^{10}$ cells, instead of the 20 μ g previously reported.

In addition to its trypsin-like activity indicated by hydrolysis of Z-Lys-SBzl, the protease purified by Superose 12 had a chymotrypsin-like specificity revealed by the hydrolysis of Suc-Ala-Ala-Pro-Phe-SBzl. Both of these activities were inhibited by STI. Any possibility that a multicatalytic proteinase was responsible for this dual activity was eliminated by adding Z-Lys-SBzl to the enzyme assayed, with a sufficient amount of Suc-Ala-Ala-Pro-Phe-SBzl to reach the maximal velocity (7 K_m), and the residual activity was measured. No increase in enzymic activity was recorded after adding the second substrate, indicating that the proteinase has one active site with both trypsin and chymotrypsin-like specificities. The same active site concentration was also found upon titration with STI, using either the trypsin- or the chymotrypsin-sensitive substrate (Fig. 1). The specificity constants (k_{cat} , K_m) were also determined for each sub-

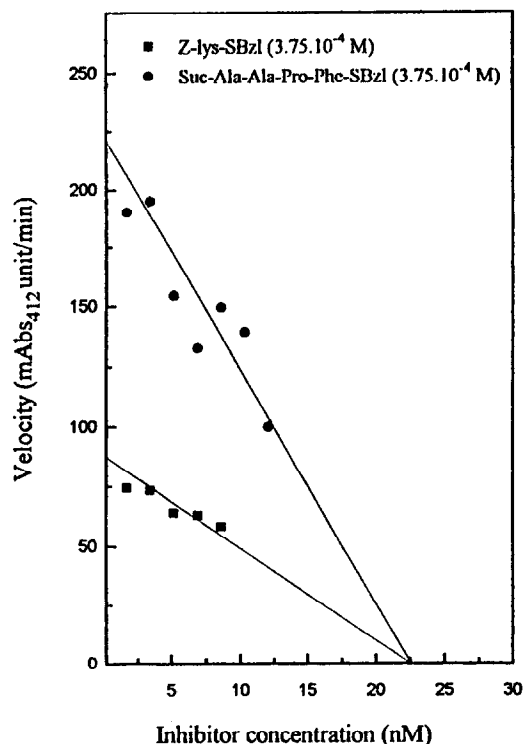


Fig. 1. Titration of the membrane-associated U-937 protease active site. Purified proteinase (5 μ l) was incubated for 15 min at 25°C with increasing amounts of STI (0 – $1.2 \cdot 10^{-8}$ M final concentration) before adding Z-Lys-SBzl or Suc-Ala-Ala-Pro-Phe-SBzl (0.37 mM final). Δ mAbs₄₁₂ was recorded for 5 min.

strate; the results show similar specificities for trypsin- and chymotrypsin-like cleavage sites (Table 1).

In keeping with these results a single sequence was found upon N-terminal sequencing of the purified material. This experiment was carried out after reverse-phase chromatography of 100 pmol protease on a C4 column eluted with H_2O /isopropanol/formic acid solution to remove the high salt content necessary to preserve the proteinase activity. The first 32 N-terminal residues were unambiguously identified (Fig. 2). This sequence was compared to the most similar sequences found in the MIPS data bank (Martinsried Institut for Protein Sequence) using the FASTA program [28]. It is the same as that of human cathepsin G [29] except at position 12

where a Val residue was found in addition to the Arg residue present in the cathepsin G sequence (Fig. 2).

The homology of the U-937 membrane proteinase with cathepsin G was further investigated by measuring the activity of commercial cathepsin G from human leukocytes towards thiobenzylester substrates, and its susceptibility to inhibitors such as benzamidine, STI and the synthetic peptide R13K, which spans the V3 loop sequence and includes the conserved GPGR sequence [13]. Both proteinases were inhibited in a similar manner by all three inhibitors (Table 2). The peculiar behaviour of the U-937 membrane proteinase, which is abnormally retained on the Superose 12 column ($M_{r,app.} < 8$ kDa) also occurred with leukocyte cathepsin G, as reported by others [30].

Both proteinases had similar antigenicity when analyzed by immuno-blotting (Fig. 3) and ELISA with anti-human cathepsin G antibodies. However, the $M_{r,app.}$ of commercial cathepsin G from human leukocytes and that of U-937 membranes were slightly different (Fig. 3). This difference was not apparent after endoglycosidase F treatment, demonstrating that the two enzymes are differently glycosylated. A sugar-dependent heterogeneity of cathepsin G has already been reported by Watorek et al. [31].

The existence of a cathepsin G-related protein in U-937 was explored using a cDNA library constructed from poly(A)⁺ RNA in λ gt11. This library was screened by PCR using two oligonucleotide pairs, taking into account the possible valyl residue at position 12. The first pair consisted of a sense 23mer (81–103) CGGGAGAG-CAGGCCCCACTCCGT and an antisense 21mer (620–600) GGGGCCTCCGGAATCCCCCTT, oligonucleotides corresponding to the conserved KGDSGGP sequence of serine proteinase. The second pair consisted of degenerate oligonucleotides corresponding to the 90–103 and the 616–603 sequences of cathepsin G [29]. PCR were carried out using Vent polymerase or its mutant deprived of exonucleotidase activity. Amplified fragments were cloned in pUC19 and double-strand sequencing was performed according to Sanger using a Sequenase kit (USB) [32]. All sequences were identical to that published for cathepsin G. No evidence was thus found for the presence of an enzyme similar to, but distinct from, cathepsin G in U-937. The presence of

Table 1

Kinetics parameters for hydrolysis of Z-Lys-SBzl and Suc-Ala-Ala-Pro-Phe-SBzl by the membrane-associated cathepsin G-like proteinase of U-937 cells and by human leukocyte cathepsin G

Substrate	Membrane U-937 cathepsin G-like				Human leukocyte cathepsin G			
	K_m (mM)	V_m (μ M \cdot s ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)	K_m (mM)	V_m (μ M \cdot s ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)
Z-Lys-SBzl	1.15	0.42	46	41	0.76	1.25	16	21
Suc-Ala-Ala-Pro-Phe-SBzl	0.26	0.16	17	68	0.51	1.15	14	28

Kinetic constants were calculated from Hanes linear plot using at least seven substrate concentrations.

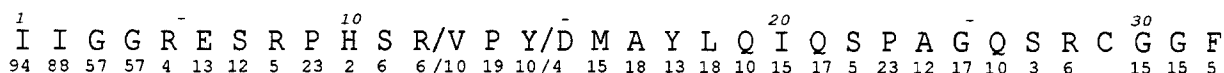


Fig. 2. Amino-terminal sequence of purified membrane-associated U-937 cathepsin G-like protease. About 100 pmol U-937 membrane-associated protease was used for N-terminal sequencing. The number of pmol per cycle is indicated below each residue. The first 32 residues were identical to those in human leukocyte cathepsin G, except at position 12 where a Val residue was found in addition to the Arg residue present in the cathepsin G sequence.

cathepsin G-like material at the surface of U-937 membranes has been further investigated using a monoclonal antibody against human leukocyte cathepsin G. Fluorescence analysis indicated that U937 cells could be stained by anti-cathepsin G mAbs at a level similar to that with anti-CD4 mAbs (Fig. 4).

4. Discussion

The proteinase we have purified and examined in this study is present at the surface of U-937 cells, from which it can be released by high salt treatment and sonication. This proteinase is strongly inhibited by recombinant gp120 by interaction with its V3 loop, and this interaction can lead to cleavage of the peptide chain [15,16]. Such a cleavage could induce a conformational change of gp120 required for subsequent virus internalization [33,34]. If so, the specificity of the enzyme should be wide enough to allow interaction or infection by HIV-1 and possibly HIV-2 strains that may infect monocytic cell lines. Cleavage sites of trypsin-, chymotrypsin- or aspartic acid-like specificity have been demonstrated at the tip of the V3 loop of all infectious HIV-1, HIV-2 and SIV isolates [8–11,16,35]. It has therefore been suggested that one multispecific proteinase or several proteinase are required to ensure cleavage of the V3 loop in all HIV strains [15]. We have demonstrated that the enzyme from U-937 cell membrane is specific for both trypsin- and chymotrypsin-like cleavage sites, which fits with the requirement for a multispecific proteinase. Moreover, the specificity constants (k_{cat} , K_m) for the tryptic and chymot-

ryptic cleavage sites are similar, indicating that the two activities might be similarly efficient. The proteinases previously reported by Kido et al. [10] and Murakami et al. [12] in Molt 4-cells, although distinct from the cathepsin G-related proteinase from U-937 cells, also have dual specificity. This is not the case, however, for dipeptidyl peptidase IV, which has a restricted specificity and is not inhibited by peptides of the V3 loop of gp120 [14].

The amino acid sequence of the first 32 N-terminal residues of the purified proteinase was the same as that of human leukocyte cathepsin G, which agrees with the chymotrypsin-like specificity of this proteinase. However, there has been no report, so far, describing a trypsin-like specificity for cathepsin G except for the cleavage of an Arg-Ser bond in the C3 component of complement [36]. This study shows that commercial leukocyte cathepsin G hydrolyses Z-Lys-SBzl as well as Suc-Ala-Ala-Pro-Phe-SBzl. Commercial cathepsin G also has the same sensitivity and specificity towards inhibitors, including the V3 loop peptide R13K, and has physicochemical properties similar to those of the U-937 enzyme. Both enzymes also share common antigenic properties.

Although initially described in the granules of polymorphonuclear leukocytes (neutrophils), cathepsin G has also been identified in U-937 promonocytic cells [29,36,37]. Its biological function is still far from clear and little is known about its physiological substrates. However, it has been reported to degrade proteoglycans efficiently [31] and generate angiotensin 2 from angiotensin 1 [38]. More recently a small fraction of U-937 cathepsin G has been found to be associated with the

Table 2
Inhibition of U-937 membrane-associated cathepsin G-like proteinase and leukocyte cathepsin G

Inhibitor	Proteinase		<i>E/I</i>	Inhibition (%)	
				Z-Lys-SBzl (0.37 mM)	Suc-Ala-Ala-Pro-Phe-SBzl (0.37 mM)
STI (17 nM)	U-937 Mb. cathepsin G-like	9 nM	0.5	82	86
	Leukocyte cathepsin G	9 nM	0.5	77	78
Benzamidine (2 mM)	U-937 Mb. cathepsin G-like	9 nM	$4.5 \cdot 10^{-6}$	24	24
	Leukocyte cathepsin G	9 nM	$4.5 \cdot 10^{-6}$	14	26
R13K (60 μ M)	U-937 Mb. cathepsin G-like	9 nM	$1.5 \cdot 10^{-4}$	75	78
	Leukocyte cathepsin G	9 nM	$1.5 \cdot 10^{-4}$	70	66

Membrane-associated cathepsin G-like proteinase from U-937 cells and cathepsin G from human leukocytes (9 nM final) were incubated with inhibitor solutions for 15 min at 25°C in 200 μ l of 0.10 M Tris-HCl buffer, pH 8.2, containing 0.35 mM 5,5'-dithiobis (nitrobenzoic acid). The reaction was started with 5 μ l of substrate (Z-Lys-SBzl or Suc-Ala-Ala-Pro-Phe-SBzl, 0.37 mM final). The absorbance at 412 nm was recorded for 5 min.

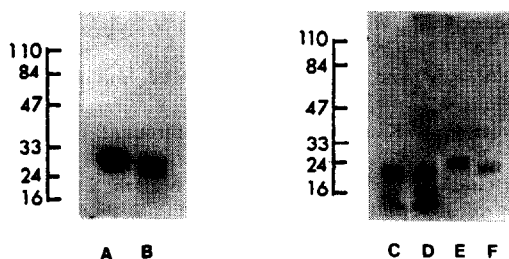


Fig. 3. Immunoblotting of purified membrane-associated U-937 cathepsin G-like protease and leucocyte cathepsin G with anti-cathepsin G antibodies. Samples (about 20 ng) of membrane-associated U-937 cathepsin G were electro-immunoblotted after SDS-PAGE without (A) and with (B) prior reduction, and before (E) and after (F) deglycosylation. Commercial cathepsin G from human leukocytes is shown for comparison, prior to (C) and after (D) deglycosylation.

plasma membrane, to participate in the generation of active fragments from the C3 component of complement, and in the inactivation of interleukin 6 together with a peptidyl-transferring enzyme [36,39]. We used a monoclonal antibody directed against human cathepsin G to confirm that the proteinase is present at the surface of U-937 cells. The location of the proteinase at the surface of the plasma membrane, together with its dual specificity for trypsin and chymotrypsin sites, make it a good candidate to participate in the entry of HIV into these cells should a proteinase be required. However, cathepsin G has not been found in other cells that are sensitive to HIV-1 infection, suggesting that other proteinases or cofactors in such cells should be involved in the interaction with the V3 loop [2,3,15]. We are now examining the interaction between U-937 membrane cathepsin G and synthetic peptides mimicking the crown of the V3 loop of HIV strains with different tropism for T lymphocytes or monocytes. Transfection of cathepsin G into cells non-permissive to HIV will also provide further evaluation of the role of this proteinase in HIV infection.

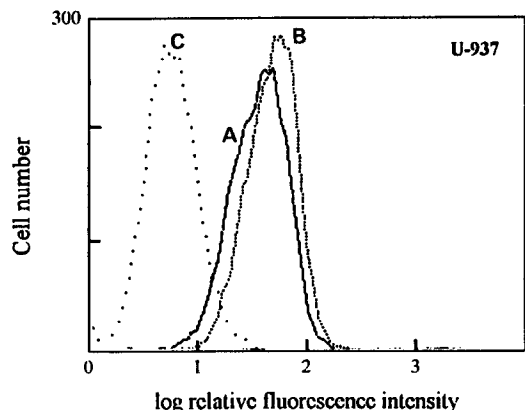


Fig. 4. Indirect fluorescence analysis of cathepsin G expression at the surface of U-937 cells. Fluorescence intensity is shown after cell labelling with anti-human cathepsin G (A) and anti-CD4 (B). Peak (C) denotes the fluorescence of the FITC labelled second antibody.

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